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1. Chong, M. W. et al., Int. J. Pharmaceutics (Jun 1998) 167(1-2): 25-36
2. Chen, B.-L. et al. J. Pharmaceutical Sciences (1994) 83(12): 1657-1661
3. Chen, B.L. et al. Pharmaceutical Res. (1994) 11(11): 1581-1587
4. Liu, H. et al. Mol. Cell. Biochem. (1997) 169(1&2): 43-50
5. Negro, A. et al. European J Biochemistry (1996) 241(2): 507-515

Strategies To Suppress Aggregation of Recombinant Keratinocyte Growth Factor during Liquid Formulation Development†

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Abstract □ Recombinant human keratinocyte growth factor (rhKGF) is a fairly unstable protein, posing a challenging problem for long-term storage. During storage, the protein unfolds at relatively low temperatures and the unfolded proteins aggregate rapidly, leading to the formation of large visible precipitates. Thermal unfolding of rhKGF displays a similar pattern, i.e., unfolding is followed immediately by aggregation as the temperature is increased. As the unfolding and aggregation (precipitation) of rhKGF limit the storage life of the protein, a search for stabilizers to suppress rhKGF unfolding and aggregation has been done by examining the effects of excipients on thermal melting temperature and on the rate of protein aggregation during storage. Sulfated polysaccharides and citrate are found to be effective in increasing the melting temperature of rhKGF or preventing its aggregation. In particular, 0.5% (w/v) heparin and high molecular weight dextran sulfate, and 0.5 M citrate are highly effective, decreasing the rates of rhKGF aggregation by about 50-fold. Other negatively charged small ions, such as phosphate, also have moderate stabilizing effects on rhKGF. A mechanistic study of the aggregation pathway of rhKGF has led to a better understanding of the stabilizing effects of these molecules. Molecules which enhance rhKGF conformational stability are capable of effectively suppressing rhKGF aggregation.

Introduction

Fibroblast growth factors (FGF) are potent mitogens for a variety of cells from mesenchymal origin and play an important role in promoting wound healing and angiogenesis.[1-5] Keratinocyte growth factor (KGF) is one of nine currently identified members of the FGF family, having proliferative and differentiating effects specifically on epithelial cells including keratinocytes.[6,7] This is in contrast to other members of the FGF family that do not have this effect. Thus, KGF may prove useful as a therapeutic agent in the treatment of diseases of and injuries to epithelial tissues.

FGF proteins often undergo rapid aggregation at elevated temperatures.[4] Acidic FGF shows physical instability at physiological temperatures and unfolds in the absence of protecting agents such as heparin.[8,9] This aggregation property, which is shared among the FGF family members, is probably due to either the specific hydrophobic nature of the unfolded polypeptide chains of these proteins or labile folding/unfolding intermediates that lead to off-pathway products.[10] This instability limits the shelf life of these proteins and creates challenges for formulation development.

Cloned and expressed in *Escherichia coli*, recombinant human keratinocyte growth factor (rhKGF) contains 163 amino acid residues.[6,7,11,12] The native protein is made of a single polypeptide chain and contains two disulfides and a buried cysteine residue. Slow aggregation, observed during purification and storage of the protein, becomes a major obstacle to obtaining a long shelf life for rhKGF in solution. Liquid formulation work has been focused on elucidation of the aggregation mechanisms and on searching for stabilizers to prevent rhKGF aggregation.

Experimental Section

Materials—Recombinant human KGF was prepared as described previously.[13,14] The rhKGF preparation used had a purity >95%, eluting as a single peak on reverse-phase, ion-exchange, and size-exclusion high performance liquid chromatography (HPLC).

All chemicals used were of analytical grade. Heparin, dextran, dextran sulfate, heparan sulfate, pentosan polysulfate, chondroitin sulfate C, and D-glucose 6-sulfate were obtained from Sigma Chemical Co., St. Louis, MO, and sucrose octasulfate was obtained from Toronto Research Chemicals Inc., Downsview, Ontario, Canada. Dulbecco's phosphate-buffered saline (D-PBS) was obtained from Gibco BRL Life Technologies Inc., Gaithersburg, MD. High-purity water and acetonitrile (Burdick and Jackson Brand) were obtained from Baxter Diagnostics Inc., Muskegon, MI. High performance liquid chromatography/Spectro grade trifluoroacetic acid (TFA) and sequanal grade 8.0 M guanidine hydrochloride (GdnHCl) were obtained from Pierce, Rockford, IL.

In Vitro Mitogenic Assay—The *in vitro* mitogenic assay was performed using a mouse epidermal keratinocyte cell line, Balb/MK, essentially according to the method of Falco *et al.* (1988).[15]

Circular Dichroism (CD) Spectra—For CD analysis, 0.5 mg/mL rhKGF was prepared either in D-PBS or in 6 M GdnHCl by simply mixing a concentrated protein stock solution with D-PBS or the 8 M GdnHCl solution. Far-UV CD spectra of these two protein samples were obtained with a Jasco-720 spectropolarimeter (Jasco Inc., Easton, MD) using a 1-mm pathlength cylindrical quartz cell.

Thermal Unfolding by Circular Dichroism—Samples of rhKGF in D-PBS at 0.1 mg/mL were loaded into a 10-mm pathlength quartz cell with a magnetic stirring bar. The cell was then placed in a Jasco JTC-343 Peltier cell holder located in the Jasco-720 spectropolarimeter. The CD signals and high-tension voltages of the photomultiplier were recorded as a function of increasing temperature, all using Jasco software and a PC computer. The difference between the temperature shown on the temperature control program and the temperature of the sample solution inside the cell was calibrated by a Model 8502-10 portable thermometer equipped with a YSI 427 thermistor probe purchased from Cole-Parmer Instrument Co., Niles, IL. Figure 2 uses uncorrected temperatures, and Figure 3 and Table 1 use corrected temperatures. In both Figures 2B and 3B, the high-tension voltage has been converted to absorbance by the JTC-340 program after correcting for the buffer contribution.

The apparent thermal melting temperature ($T_{m,app}$) was determined by finding a midpoint temperature between the native (linear interpolation of the native region) and the denatured form (either the lowest point or linear interpolation of the unfolded region) on thermal unfolding curves. The initial temperature at which the thermal unfolding transition occurs (T_{us}) was also determined as a cross point between linear interpolations of the native region and the unfolding transition region from thermal unfolding curves.

Thermal Unfolding by DSC and UV/vis—The thermal unfolding of selected samples was examined by differential scanning calorimetry (DSC) and UV/vis spectroscopy. The unfolding thermogram was determined on a Hart DSC (Hart Scientific, Pleasant Grove, UT) using 0.52 mL of a 0.5 mg/mL rhKGF solution in 20 mM sodium phosphate

† This work was presented at the 1994 ACS Symposium on Aggregation Issues in Pharmaceutical Protein Formulations, held at the National Meeting in San Diego, CA, March 1994.
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Table 1—Effect of Sulfated and Unsulfated Polysaccharides on Thermal Unfolding of rhKGF

Compound (5 mg/mL)	$\Delta T_{m,app}$[a] (CD) (°C)	ΔT_{us}[a] (CD) (°C)	ΔT_{ex}[b] (DSC) (°C)	ΔT_{is}[b] (UV/vis) (°C)
D-Glucose 6-sulfate	+1	0		
Sucrose Octasulfate	+6	+5	+9	+3
Heparin[c]	+11	+11	+8	+15
Heparin (MW 6600)	+10	+10		
Heparin (MW 3400)	+10	+9		
Heparan Sulfate[d]	+11	+12		+6
Dextran sulfate (MW 500 000)	+11	+11	+14	+16
Dextran sulfate (MW 50 000)	+11	+10	+10	
Dextran sulfate (MW 15 000)			+5	
Dextran sulfate (MW 8000)	+10	+8	+5	+9
Dextran sulfate (MW 5000)	+8	+6		
Pentosan polysulfate	+10	+10	+8	+14
Chondroitin sulfate C	+9	+9		+6
Dextran (MW 515 000)				0
Dextran (MW 39 100)	0	0		0

[a] 0.1 mg/mL rhKGF in D-PBS. [b] 0.5 mg/mL rhKGF in 20 mM sodium phosphate, 150 mM NaCl, pH 7.0. [c] Heparin had an average molecular weight of 18 000 Da. [d] 3.26 mg/mL was used for CD; 0.5 mg/mL for UV/vis absorbance.

Table 2—Half-lives for Loss of Soluble rhKGF upon 37 °C Storage in Various Formulations As Examined by IEX-HPLC

Formulation (all at pH 7)	$t_{1/2}$ at 37 °C (day)
10 mM Sodium phosphate/ 140 mM NaCl	1.8
10 mM Sodium phosphate/5% (w/v) sorbitol	1.6
10 mM Sodium phosphate/9% (w/v) sucrose	1.7
10 mM Sodium phosphate/ 100 mM NaCl/0.4% (w/v) L-aspartic acid	2.6
10 mM Sodium phosphate/ 100 mM NaCl/0.7% (w/v) L-glutamic acid	2.4
100 mM Sodium phosphate	15
100 mM Sodium citrate	25
500 mM Sodium citrate	88
10 mM Sodium phosphate/140 mM NaCl/0.5% (w/v) heparin	105

and 150 mM NaCl at pH 7 and a heating rate of 60 °C/h. The results were converted to heat capacity data and corrected for the blank (obtained from a second heating cycle of the same samples; rhKGF precipitates during the initial run, leaving only buffer for the second run) with the Hart software. The midpoint of the aggregation exotherm was designated as T_{ex} and was used to determine the stability of the rhKGF in the various formulations tested. The increase in light scattering upon unfolding and/or aggregation was followed by absorbance at 287 nm with heating in a Gilson Response II spectrophotometer, equipped with a Peltier thermal cuvette holder (Ciba Corning, Pleasanton, CA). The rhKGF at concentrations of 0.5 mg/mL prepared in 20 mM sodium phosphate and 150 mM NaCl at pH 7 was analyzed in thermal cuvettes with a pathlength of 10 mm and heating rates of 0.5 °C per reading. A typical experiment covering 60 °C took about 3 h. The temperature at which the absorbance begins to increase was designated as T_{is} and was applied to detect starting of the rhKGF unfolding and/or aggregation for various formulations.

Formulation Setup and Sample Analysis—Formulations and protein analysis (ion-exchange HPLC, size-exclusion HPLC, UV/vis absorbance, and SDS–PAGE) were described elsewhere,[14] with the exception that the ion-exchange and size-exclusion HPLC methods were run on a Hewlett-Packard (HP) 1050 liquid chromatography system (Hewlett-Packard Co., Palo Alto, CA). The osmolarities of formulation samples were measured by a Model 5500 vapor pressure osmometer from Wescor Co. (Logan, UT).

Reverse-phase HPLC (RP-HPLC) analysis of rhKGF samples was performed using an HP 1090 liquid chromatography system equipped with an HP 3D Chemstation for data acquisition. Absorbance at 220 nm was monitored on an HP diode-array detector. Injected samples were first equilibrated for 5 min in 75% buffer A [0.1% (v/v) TFA/water] and 25% buffer B [90% (v/v) acetonitrile and 0.1% (v/v) TFA/water] in a Vydac TP 214 C4 column (250 mm × 4.6 mm) purchased from Alltech Associates, Inc. (Deerfield, IL). The protein was subsequently eluted using step gradients at a flow rate of 0.7 mL/min, first from 25% buffer B to 35% buffer B in 10 min, then from 35% to 50% in 30 min, and finally from 50% to 95% in 10 min.

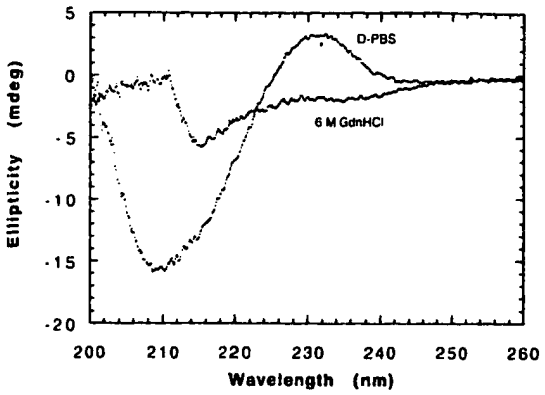

Figure 1—Far-UV CD spectra of native (in D-PBS) and unfolded (in 6 M GdnHCl) rhKGF.

Quantification of Soluble rhKGF—Soluble rhKGF was quantified by absorbance measurements at 280 nm. Samples were centrifuged at 15000*g* for 5 min and the supernatants collected for absorbance measurements. Soluble rhKGF was also quantified by HPLC methods. Samples were first filtered through 0.22 μm Costar Spin-X filter units (Cambridge, CA), which yielded clear filtrates, indicating that precipitates had been filtered out. The filtrates were loaded onto the HPLC columns, and the HPLC peak area corresponding to the monomeric form was integrated. Kinetic parameters, such as rate constants and half-lives for loss of soluble monomeric rhKGF upon incubation at elevated temperatures, were determined by performing a single exponential fitting to kinetic curves of the integrated peak area versus the storage period.

Results and Discussion

Thermal Unfolding of rhKGF—Recombinant hKGF is a relatively unstable protein and undergoes rapid aggregation at elevated temperatures. A sample containing 0.5 mg/mL rhKGF prepared in an isotonic phosphate-buffered solution shows that the half-life of soluble rhKGF, as measured from its disappearance, is less than 2 days at 37 °C (Table 2). The rapid aggregation of rhKGF at elevated temperatures may be a consequence of its conformational instability.

The thermal unfolding of rhKGF prepared in D-PBS was studied by far-UV circular dichroism (CD). Figure 1 shows CD spectra for the native (in D-PBS) and the unfolded (in 6 M GdnHCl) forms of rhKGF. Since thermal unfolding of rhKGF was accompanied by aggregation and precipitation, it was not possible to obtain a CD spectrum for the thermally unfolded rhKGF. Instead, the CD spectrum for the unfolded rhKGF was measured in 6 M GdnHCl. The CD spectrum of the native rhKGF in D-PBS shows a positive peak at 230 nm, most likely reflecting the tertiary structure associated with ring stacking in β-turns or loops.[16] This 230 nm peak decreases with increasing GdnHCl concentration (Chen, B.-L.; Arakawa, T., unpublished results) and disappears upon complete unfolding by 6 M GdnHCl. Therefore it was used to monitor the conformational change during thermal unfolding of rhKGF.

(1) Thermal Unfolding of rhKGF in D-PBS is Not an Equilibrium Process between the Native and Unfolded

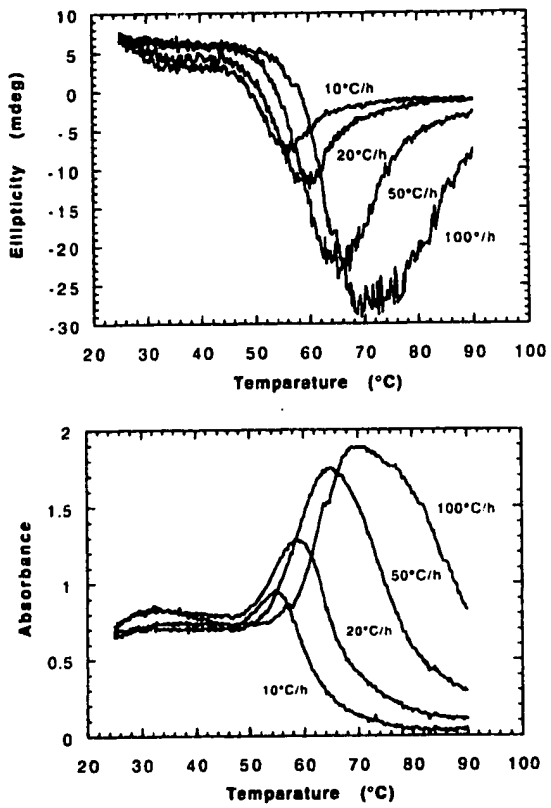

Figure 2—Thermal unfolding of rhKGF at 0.1 mg/mL in D-PBS with varying temperature-ramping rates measured by circular dichroism (ellipticity) (A) and absorbance (B) at 230 nm. Four unfolding experiments were carried out at temperature-ramping rates of 10, 20, 50, and 100 °C/h, respectively.

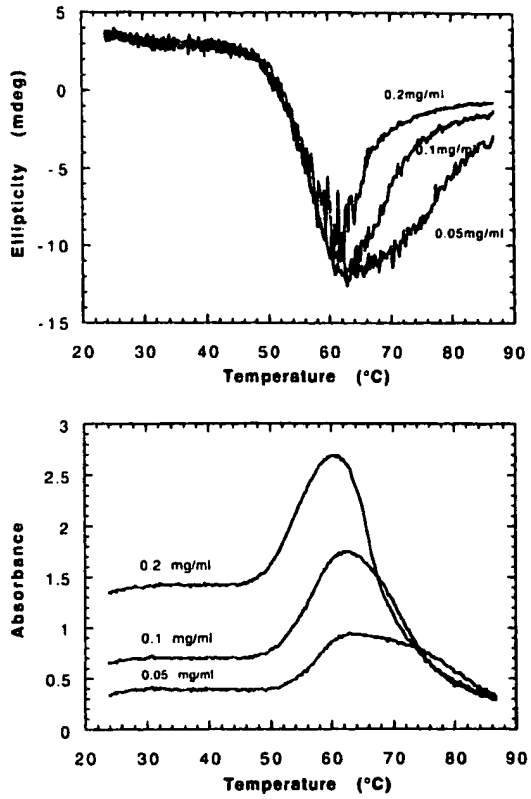

Figure 3—Thermal unfolding of rhKGF with varying protein concentrations measured by circular dichroism (ellipticity) (A) and absorbance (B) at 230 nm at a fixed temperature-ramping rate of 50 °C/h. Three thermal unfolding curves are for protein concentrations of 0.2, 0.1, and 0.05 mg/mL, respectively. CD signals of 0.2 and 0.1 mg/mL rhKGF samples were normalized to the 0.05 mg/mL rhKGF sample for comparison.

Proteins—The thermal unfolding of 0.1 mg/mL rhKGF prepared in D-PBS was followed at different temperature ramping rates. Figure 2 shows four thermal unfolding curves at temperature ramping rates of 100, 50, 20, and 10 °C/h, respectively. Thermal unfolding was followed by two signals, ellipticity and absorbance, which were simultaneously detected from the same CD instrument, converted from the high-tension voltage of the photomultiplier at 230 nm. As shown in Figure 2A, the CD signal of the native rhKGF first decreases slightly with increasing temperature, indicating a dependence of the native CD signal on temperature. A sharp decrease in the CD intensity subsequently occurs around 46–52 °C (depending on the temperature ramping rate), indicating protein unfolding. The unfolding is accompanied by a sharp increase in absorbance (Figure 2B), reflecting light scattering due to protein aggregation. There is a noticeable temperature lag between the temperatures at which unfolding starts and th temperatures at which light scattering starts to increase. For example, at the 50 °C/h temperature-ramping rate, the initial temperature for unfolding was 50 °C, while the initial temperature for light scattering was 52 °C. As the temperature is further increased, the loss of CD signals and concomitant decrease of the absorbance occur, reflecting precipitation.

Figure 2 illustrates that when the temperature-ramping rate was decreased, the unfolding was shifted to a lower temperature. This is similar to the results obtained by Sanchez-Ruiz *et al.* during their thermal unfolding study of thermolysin by differential scanning calorimetry.[17] They have found that the thermal melting temperature of thermolysin depends strongly on the scan rate, suggesting that the denaturation process is kinetically controlled. This was suggested because equilibrium was not reached between the native and unfolded forms, either due to the rate of unfolding being slow relative to the scan rate used, or because the subsequent aggregation reaction shifted the equilibrium toward the denatured state, or both. Extensive aggregati n appears to follow denaturation of rhKGF (after a temperature lag or a time lag) as manifested by a sharp increase in light scattering of the solution.

(2) Thermal Unfolding of rhKGF in D-PBS is Not a Reversible Process—All samples in the varying-temperature ramping-rate experiments showed visible precipitates after thermal unfolding and exhibited no refolding after cooling. Reversibility of thermal unfolding of rhKGF was examined at a fixed-temperature ramping rate 50 °C/h, but with varying protein concentrations of 0.05, 0.1, and 0.2 mg/mL prepared in D-PBS. Thermal unfolding curves of rhKGF at these three protein concentrations are shown in Figure 3. Upon increasing temperature, rhKGF first unfolds with a sharp decrease in CD signal at 230 nm (Figure 3A). The initial unfolding temperature appears independent of protein concentration and this may suggest that the rate-limiting step is unfolding, i.e., slow unfolding, rather than aggregation. After a small lag (probably a time lag rather than a temperature lag), an increase in light scattering is observed (Figure 2B). The temperature at which light scattering occurs depends apparently on the rhKGF concentration in the sample. Light scattering occurs at higher temperatures in samples which contain lower rhKGF concentrations. Finally, loss of the CD signal corresponding to protein precipitating out f solution (visible precipitates) was observed.

The observation of a lag between unfolding (m nitored by CD) and aggregation (monitored by increase in absorbance

converted from the high-tension voltage of the photomultiplier of the CD instrument) is due either to the fact that light scattering depends strongly on the size of the aggregates formed or to the presence of a nucleation step in forming large soluble aggregates. A lag is often observed for polymerization of proteins into large biological structures.[18] For example, a time delay was observed as a nucleation step in the aggregation of deoxyhemoglobin S to form long fibers.[19]

Reversibility was also examined by cooling down the thermally denatured rhKGF samples to temperatures below the thermal-unfolding transition region, where little or no refolding was observed. When the cooled samples were examined by HPLC methods (ion-exchange, size-exclusion, and reverse-phase) after passing through 0.22 μm Costar Spin-X filter units, an rhKGF peak was not observed. The native rhKGF had been converted to precipitates, rendering the thermal unfolding of rhKGF irreversible.

In addition to CD, DSC and UV/vis absorbance were applied to follow thermal unfolding of rhKGF. These experiments were carried out at a higher rhKGF concentration (0.5 mg/mL) in 20 mM sodium phosphate and 150 mM NaCl at pH 7. The differential scanning calorimetry thermal-melting profile of rhKGF showed mainly an aggregation exothermic peak which apparently overwhelmed the unfolding endothermic peak at this protein concentration. A second thermal scan after cooling showed no thermogram, confirming the irreversibility of the thermal unfolding.

The UV/vis absorbance trace of rhKGF thermal unfolding showed an abrupt increase at a specific temperature upon heating, indicating that light scattering occurs due to aggregation. The sharp increase in absorbance at a specific temperature suggests that aggregation may follow a conformational change. Otherwise, a gradual increase in light scattering upon heating would be expected. Wetzel *et al.* have found that the rate constant for thermal inactivation of T4 lysozyme was greatly increased at the temperature at which the protein underwent thermal unfolding.[20] It is possible that unfolding or partial unfolding of rhKGF accelerates the aggregation rate.

The thermal unfolding results suggest that rhKGF undergoes slow unfolding at elevated temperatures. The unfolded or partially unfolded proteins aggregate immediately after denaturation and these aggregates subsequently form insoluble precipitates. This aggregation/precipitation pathway correlates well with what we have observed from a detailed kinetic characterization of the aggregation mechanism of rhKGF in solutions at 37 °C by HPLC and SDS–PAGE.[14] Kinetic experiments show that the folded monomer of rhKGF slowly disappears with time and that soluble aggregates are formed. No unfolded monomer could be detected by any one of three types of HPLC (ion-exchange, size-exclusion, and reverse-phase). These aggregates are converted to highly cross-linked precipitates. Thus, both thermal unfolding and aggregation kinetic studies suggest that unfolding is the initial step in the aggregation/precipitation pathway. It is possible that unfolding intermediates instead of the fully unfolded form may trigger aggregation.[21] In fact, Dabora et al. have suggested the presence of an early intermediate in their study of refolding kinetics of aFGF, a homologous protein to KGF.[8] Kinetic experiments examining the presence of unfolding intermediates of rhKGF are underway (Chen, B.-L.; Arakawa, T., unpublished results). However, it is advantageous to prevent aggregation at the initial unfolding stage, whether or not aggregation starts from fully unfolded form or partially unfolded intermediates. Thus we have focused our search for stabilizers that can enhance conformational stability of rhKGF.

Effects of Various Additives on rhKGF Stability—*(1) Heparin and Sulfated Polysaccharides*—Heparin and its analogous polysaccharides, such as heparan sulfate, dextran sulfate, pentosan polysulfate, and chondroitin sulfate, have been found to enhance the stability of acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) and to prevent protein aggregation of aFGF[9,22] and bFGF.[23] The effects of these polysaccharides on the conformational stability of rhKGF were examined. We have included two highly sulfated mono- and disaccharides and two unsulfated polysaccharides for comparison. Table 1 shows the difference in apparent melting temperature of rhKGF, $\Delta T_{m,app}$, in the absence and presence of these compounds measured by CD using a temperature-ramping rate of 50 °C/h. Since aggregation/precipitation may affect determination of the thermal melting temperature, the temperature at which unfolding starts (T_{us}) was also determined, as it is relatively less affected by aggregation/precipitation. The difference in T_{us} is listed in Table 1. In most cases, it seems that the observed difference in stability expressed by $T_{m,app}$ correlates well with that by T_{us}.

The differences in the temperatures determined from the maximum of the exothermic peak on DSC thermogram (T_{ex}) and from the initial increase in the absorbance at 287 nm (T_{ls}) are also included in Table 1. Although T_{ex} and T_{ls} associate with the aggregation event and $T_{m,app}$ and T_{us} are related to the unfolding transition, they correlate with one another in terms of the relative ability of the additives to increase the stability of rhKGF because of the coupling between unfolding and aggregation. Their absolute values depend on the protein concentrations and techniques used, because of the complexity of the unfolding process.

Among the compounds tested, a dextran shows no increase in $T_{m,app}$, indicating no stabilization. D-Glucose 6-sulfate shows a marginal stabilization effect as it increases $T_{m,app}$ of the protein by only 1 °C. Sucrose octasulfate shows a moderate effect, increasing $T_{m,app}$ by 6 °C and sulfated polysaccharides, such as heparin, dextran sulfate, pentosan polysulfate, and chondroitin sulfate C, all show a substantial increase in $T_{m,app}$.

$\Delta T_{m,app}$ appears to depend on the molecular weight of these polysaccharides (or the number of sugar units). For example, an increase in heparin molecular weight from about 3400 to 18 000 Da increases $\Delta T_{m,app}$ by 1 °C. Similarly, an increase in dextran sulfate molecular weight from 5000 to 500 000 Da results in about a 3 °C increase in $\Delta T_{m,app}$. This probably has something to do with the binding affinity of these compounds to rhKGF as is implied in the case of aFGF.[9,24] The increase from short-chain saccharides to long-chain saccharides may increase the binding affinity for the protein and is thermodynamically more favored.

Heparin, heparan sulfate, and dextran sulfate with a molecular weight of 500 000 Da can increase $T_{m,app}$ by more than 10 °C according to all of the techniques used and significantly enhance the conformational stability of the protein. To investigate the ability of sulfated polysaccharides to suppress rhKGF aggregation under accelerated stability testing, solutions containing 0.5 mg/mL rhKGF were prepared in 0.5% (w/v) heparin, 10 mM sodium phosphate, and 140 mM NaCl at pH 7. A control was prepared without heparin. The amounts of soluble rhKGF in these two samples after incubation at elevated temperatures were quantified by IEX-HPLC. At 37 °C, the half-life for the loss of soluble rhKGF was 105 days with heparin and 1.8 days without heparin (Table 2). Addition of 0.5% heparin increases the half-life of soluble rhKGF by about 60-fold.

Stabilizers examined in Table 1 all have common properties, i.e., they all contain negative charges. Electrostatic interactions between these stabilizers and rhKGF may play a critical role in the stabilization of rhKGF, which is a very basic protein. These compounds may stabilize rhKGF by interact-

ing with positively charged clusters on the surface of the native protein, thus decreasing the electrostatic repulsion between positively charged residues in these clusters.[14]

(2) Citrate and Other Stabilizers—In a previous report, rhKGF has shown maximum stability at neutral pH.[14] The rate of aggregation is slower at neutral pH than at either acidic or basic pHs. Protein solutions containing 0.5 mg/mL rhKGF were prepared in various formulations as listed in Table 2 and stored at 37 °C. The amount of soluble rhKGF in stored samples were analyzed by IEX-HPLC and showed first-order decay kinetics. Half-lives for the loss of soluble rhKGF for different formulations were determined by performing a single expenential fitting to kinetic curves of the remaining soluble rhKGF versus storage time and are shown in Table 2.

At 10 mM sodium phosphate, formulations containing 5% (w/v) sorbitol or 9% (w/v) sucrose or 140 mM NaCl show about 1.6–1.8 day half-lives. These three formulations have similar osmolarities (282, 304, and 304 mmol/kg for formulations containing NaCl, sucrose, and sorbitol, respectively), but different ionic strengths. It appears that the difference in ionic strength in these three samples plays a minor role in the stabilization of rhKGF. The addition of 0.4% (w/v) L-aspartic acid or 0.7% (w/v) L-glutamic acid to formulations containing 10 mM sodium phosphate and 100 mM NaCl increases the half-lives to 2.4–2.6 days. Amino acids with acidic side chains appear to have small stabilizing effects on rhKGF. The resulting increase in half-life is less than 2-fold. On the other hand, phosphate appears to be a moderate stabilizer for rhKGF. The half-life can be increased by 8.3-fold by increasing the phosphate concentration from 10 to 100 mM.

Among the small ions tested, sodium citrate was the most effective in stabilizing rhKGF. At 100 mM, phosphate had a half-life of 15 days and citrate had a half-life of 25 days for the loss of soluble rhKGF. As discussed in a previous report,[14] this may be as a result of the increased number of negatively charged groups in citrate ions than phosphate ions at neutral pH. It is possible that more negative charges, as well as the geometric arrangement of these charges, make citrate interact better with the positively charged residues on rhKGF, which results in better stabilization of the native conformation of the protein. During a separate thermal unfolding experiment, 50 mM sodium citrate showed a 3 °C increase in the thermal melting temperature of rhKGF as compared with that of D-PBS. This is consistent with our hypothesis of the mechanism of conformational stabilization by citrate.

Further increase in the citrate concentration to 500 mM increases the half-life for preserving soluble rhKGF to 88 days. This is a 50-fold increase compared with the phosphate-buffered saline formulation (1.8 days). The effect of citrate on preserving the native rhKGF conformation upon storage at elevated temperatures was examined by the *in vitro* mitogenic bioassay. Figure 4 shows the percentage of the *in vitro* bioactivity of rhKGF as a function of citrate concentration after incubation at 37 °C for 6 months. The rhKGF bioactivity increases with increasing citrate concentration in the formulations, demonstrating that preservation of the bioactivity of rhKGF also depends on citrate concentration.

Both heparin and citrate are able to prevent rhKGF aggregation to a great extent. They both stabilize rhKGF conformationally and prevent rhKGF aggregation at very early stages on the aggregation pathway. They should be very useful in the formulation of rhKGF. Heparin is already known to stabilize other members of the FGF family. If electrostatic interaction plays a key role in stabilizing the FGF family members, citrate could be a good stabilizer for ther FGF family members as well.

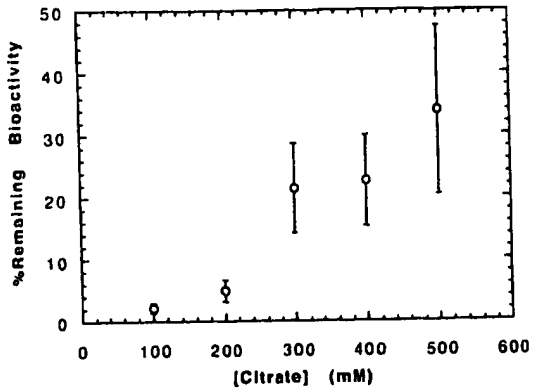

Figure 4—Dependence of *in vitro* bioactivity of rhKGF samples on citrate concentration. rhKGF samples at 0.5 mg/mL prepared in 100–500 mM sodium citrate were incubated at 37 °C for 6 months and then assayed for *in vitro* mitogenesis.

References and Notes

1. Esch, F.; Baird, A.; Ling, N.; Ueno, N.; Hill, F.; Denoroy, L.; Klepper, R.; Gospodarowicz, D.; Bohlen, P.; Guillemin, R. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 6507–6511.
2. Gospodarowicz, D.; Cheng, J.; Lui, G.-M.; Baird, A.; Bohlen, P. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 6963–6967.
3. Thomas, K. A.; Rios-Candelore, M.; Gimenez-Gallego, G.; DiSalvo, J.; Bennett, C.; Rodkey, J.; Fitzpatrick, S. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 6409–6413.
4. Burgess, W. H.; Maciag, T. *Annu. Rev. Biochem.* **1989**, *58*, 575–606.
5. Baird, A.; Klagsbrun, M. *Cancer Cells* **1991**, *3*, 239–244.
6. Rubin, J. S.; Osada, H.; Finch, P. W.; Taylor, W. G.; Rudikoff, S.; Aaronson, S. A. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 802–806.
7. Finch, P. W.; Rubin, J. S.; Miki, T.; Ron, D.; Aaronson, S. A. *Science* **1989**, *245*, 752–755.
8. Dabora, J. M.; Sanyal, G.; Middaugh, C. R. *J. Biol. Chem.* **1991**, *266*, 23637–23640.
9. Volkin, D. B.; Tsai, P. K.; Dabora, J. M.; Gress, J. O.; Burke, C. J.; Linhardt, R. J.; Middaugh, C. R. *Arch. Biochem. Biophys.* **1993**, *300*, 30–41.
10. King, J. *Chem. Eng. News* **1989**, *67*, 32–54.
11. Ron, D.; Bottaro, D. P.; Finch, P. W.; Morris, D.; Rubin, J. S.; Aaronson, S. A. *J. Biol. Chem.* **1993**, *268*, 2984–2988.
12. Yan, G.; Nikolaropoulos, S.; Wang, F.; Mckeehan, W. L. *In Vitro Cell. Dev. Biol.* **1991**, *27A*, 437–438.
13. Burnette, W.; Mar, V.; Cieplak, W.; Morris, C.; Kaljot, K.; Marchitto, K.; Sachdev, R.; Locht, V.; Keith, J. *Bio/Technol.* **1988**, *6*, 699–706.
14. Chen, B.-L.; Arakawa, T.; Morris, C. F.; Kenney, W. C.; Wells, C. M.; Pitt, C. G. *Pharm. Res.* **1994**, *11*, 1582–1589.
15. Falco, J. P.; Taylor, W. G.; Di Fiore, P. P.; Weissman, B. E.; Aaronson, S. A. *Oncogene* **1988**, *2*, 573–578.
16. Narhi, L. O.; Rosenfeld, R.; Wen, J.; Arakawa, T.; Prestrelski, S. J.; Philo, J. *Biochemistry* **1993**, *32*, 10819–10825.
17. Sanchez-Ruiz, J. M.; Lopez-Lacomba, J. L.; Cortijo, M.; Mateo, P. L. *Biochemistry* **1988**, *27*, 1648–1652.
18. Oosawa, F.; Asakura, S. *Thermodynamics of the Polymerization of Protein*; Academic Press: New York, 1975; Chapter 4.
19. Hofrichter, J.; Ross, P. D.; Eaton, W. A. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4864–4868.
20. Wetzel, R.; Perry, L. J.; Mulkerrin, M. G.; Randall, M. In *Protein Design and the Development of New Therapeutics and Vaccines*; Poste, G.; Hook, J. B., Eds.; Plenum Press: New York, 1990; pp 79–115.
21. Mitraki, A.; King, J. *Bio/Technology* **1989**, *7*, 690–697.
22. Tsai, P. K.; Volkin, D. B.; Dabora, J. M.; Thompson, K. C.; Bruner, M. W.; Gress, J. O.; Matuszewska, B.; Keogan, M.; Bondi, J. V.; Middaugh, C. R. *Pharm. Res.* **1993**, *10*, 649–659.
23. Kajio, T.; Kawahara, K.; Kato, K. *FEBS* **1992**, *306*, 243–246.
24. Mach, H.; Volkin, D. B.; Burke, C. J.; Middaugh, C. R.; Linhardt, R. J.; Fromm, J. R.; Loganathan, D.; Mattsson, L. *Biochemistry* **1993**, *32*, 5480–5489.

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